Cetuximab-Coated Thermo-Sensitive Liposomes Loaded with Magnetic Nanoparticles and Doxorubicin for Targeted EGFR-Expressing Breast Cancer Combined Therapy

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➢ Article information

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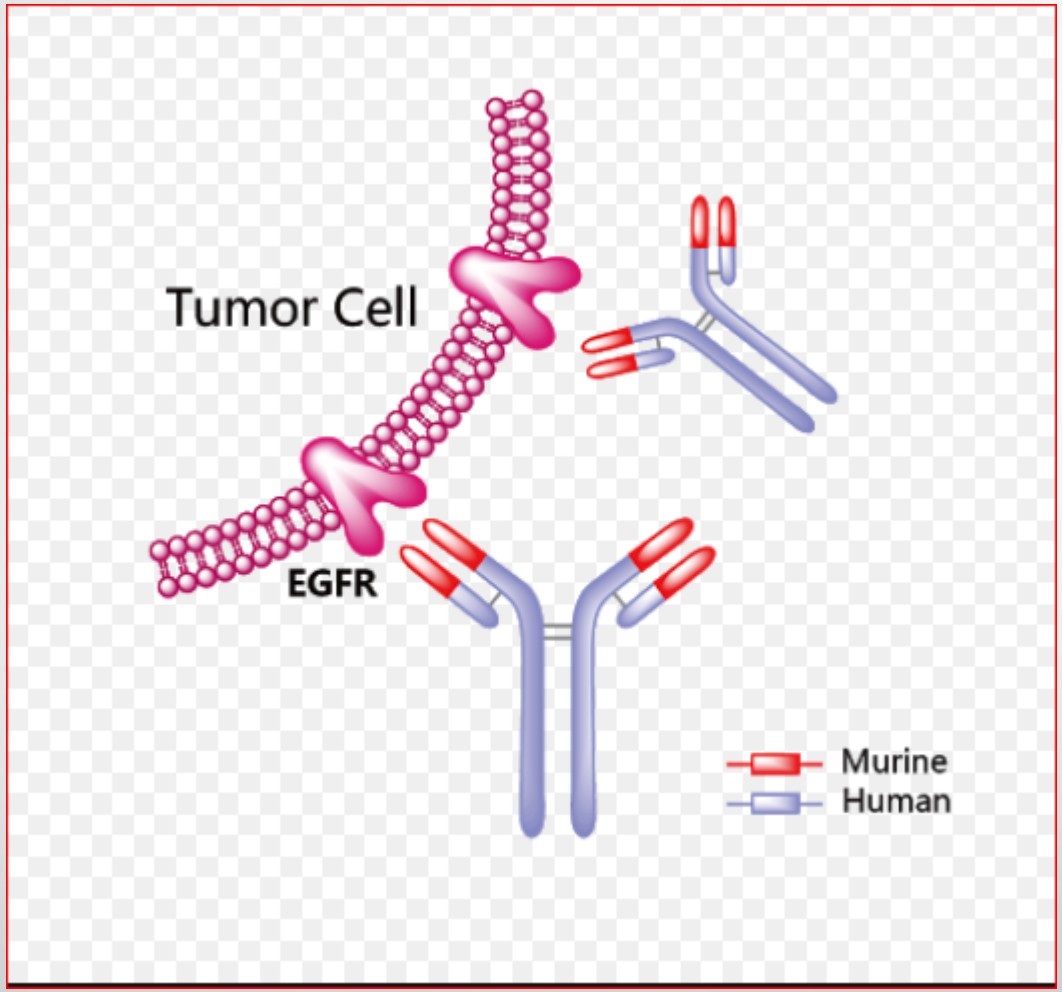
➢ Introduction

- Breast cancer is one of the most deadliest cancer types in women.
- Conventional chemotherapy and radiotherapy .
- The overexpression of the epidermal growth factor receptor (EGFR)
- Overexpression 82-90%
- Including increased cancer cell , proliferation and inhibited apoptosis.

➢ Introduction

- Cetuximab (CET) is a monoclonal antibody treatment that has been used to target EGFR signaling in cancer cells .
- Anti-cancer effects in EGFR-overexpressing tumor cells in vitro and in vivo
- Inhibit or reduce the growth of cancer cells
- CET is already approved for clinical use in head and neck cancer and colorectal cancer.



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➢ Introduction

- Doxorubicin (DOX) is one of the **most powerful chemotherapy drugs**
- Activates cell apoptosis
- kill cancer cells at every point in their life cycle
- CET and DOX is often hampered by their side effects, including **dose-dependent cardio-toxicity**.
- Their **lack of specificity** towards cancer cells, which can result in systemic cytotoxicity.

➢ Introduction

- Various novel cancer therapeutic strategies have been proposed to improve the local targeting of anti-cancer treatments to the tumor cells .
- Therapies that are responsive **to external stimuli** such as **light, magnetic field, ultrasound, and radio-frequency.**
- Magnetic nanoparticles (MNPs) have been used for targeted drug delivery in combination with external exposure to a magnetic field that enables the local delivery of therapeutic and diagnostic agents.

➢ Introduction

- Iron oxide (Fe3O4) MNPs have also been used as a delivery system for chemotherapeutics, gene therapeutics, and photo- thermal therapeutics .
- **Biocompatibility, and ease of size control**.
- Generate heat when exposed to near-infrared (NIR) laser irradiation.

➢ Introduction

- Thermo-sensitive liposomes (TSLs), release the encapsulated drug when heated to fever temperatures (~40–42°C)
- Biocompatibility, biodegradability, and loading capacity.
- Iron oxide nanoparticles exhibit excellent photo-thermal treatment efficacy when excited by NIR laser irradiation .
- Loading of these iron oxide nanoparticles into TSLs improved NIR-laser-triggered drug release.

Materials and methods

Synthesis of Citric Acid-Coated Fe_3O_4-MNPs (CMNPs)

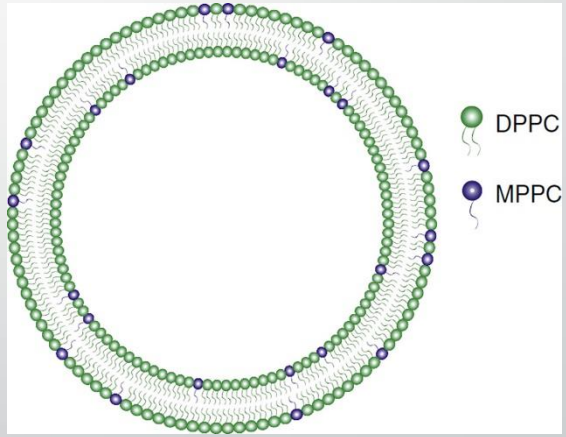
- Dissolving 0.875 g $FeCl_2$ and 2.375 g $FeCl_3$ (Fe^{2+}: Fe^{3+} = 1:2)
- In 40 mL of double-distilled water (DDI water)
- 100 rpm for 15 min at 80°C
- The stirring speed was increased to 1000 rpm and
- 5 mL NH_4OH …30 min

Coat MNPs with citric acid

- The temperature of the solution was increased to 95°C
- Citric acid was added drop by drop
- The reaction was proceed further for 90 min.
- Cooling at room temperature
- Diluted twice with DDI water and subjected to magnetic separation for 10 min
- The supernatant containing CMNPs
- Washed with DDI water in a 10 kDa molecular weight cut-off (MWCO) hollow fiber module
- Remove excess citric acid and NH4OH

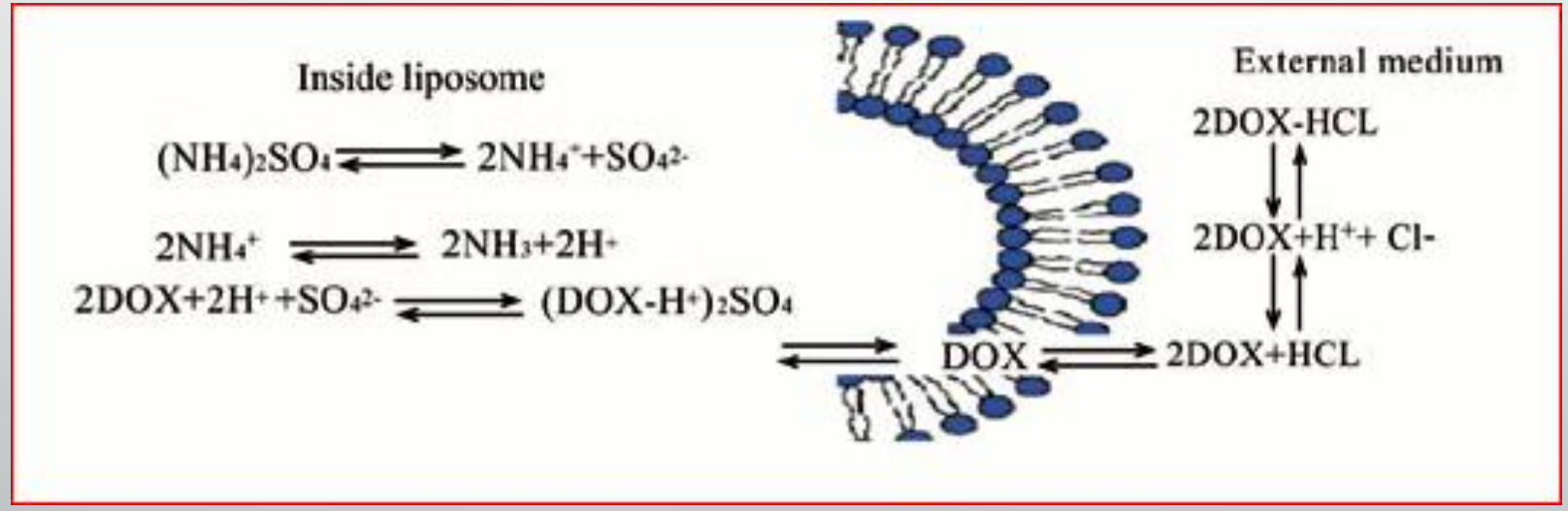
Preparation of CMNP-TSLs

- 30mg DPPC, 6 mg DSPE-mPEG-2000 and 4mg MPPC
- Dissolved in a chloroform/methanol solution (2:1 V/V).
- The organic solvent was removed using a rotary evaporator
- 39°C with a water bath for 2 h.
- Rehydrated with an ammonium sulfate
- sonicated for 15 min.
- the liposomes were extruded
- using double-stacked polycarbonate membranes filter
- The un-encapsulated CMNPs were removed by centrifugation



Preparation of DOX-Loaded CMNP-TSLs (DOX-CMNP-TSLs)

- An ammonium sulfate plus CMNP-TSLs suspension
- Ion exchange mechanism
- Ammonium ion is exchanged with the drug



Preparation of CET-Coated DOX-CMNP-TSLs (CET-DOX-CMNP-TSLs)

- 0.6mg DSPE- mPEG-COOH was added to NHS and EDC (molar ratio 1:1)
- pH 7.4 for 30 min
- 2.0 mg of CET was added at 4°C and incubated for 12 h
- Obtain CET-DSPE-mPEG-COOH
- The DOX-CMNP-TSLs solution and CET-DSPE-mPEG-COOH solution were mixed and shaken at 4°C in the dark for 5 h to obtain CET- DOX-CMNP-TSLs
- The unconjugated CET was removed by chromatography

Determination of Loading Efficiency of DOX into CMNP-TSLs

- The quantity of CMNPs and DOX in the TSLs was determined by spectrophotometry
- Using aqua regia to dissolve the sample
- Using DDI water to dilute the sample.
- The sample solution was added to sulfosalicylic acid solution.
- 1.0 mL of an NH3-NH4Cl buffer solution was added
- total volume was increased to 5 mL with the addition of DDI water
- gentle shaking and measurement of absorbance of the solution at 380–460 nm using a UV– VIS spectrophotometer

- For the content of DOX, the measurement was conducted after denaturing the emulsion with absolute alcohol
- the absorbance value at 480 nm
- The CMNPs and DOX concentrations were determined using a standard curve

$$\text{Encapsulation efficiency\%} = \left(\frac{\text{Wt of encapsulated CMNPs or DOX}}{\text{Wt of CMNP or DOX added}} \right) \times 100 \quad \text{(Equation 1)}$$

Determination of Conjugation Efficiency of CET Onto TSLs

- BCA protein assay (is used for quantitation of total protein in a sample)
- 50 parts of the BCA Reagent A with 1 part of the BCA Reagent B (50:1, Reagent A:B)
- 20 μL sample and 200 μL working agent at 37°C for 30 min
- absorbances were determined at the wavelength of 570 nm
- Unconjugated CET was removed by chromatography

$$\text{Conjugation efficiency} = \left(1 - \frac{\text{Cfree} * \text{V1}}{m_0}\right) \times 100\% \quad \text{(Equation 2)}$$

Characterization of CMNPs, TSLs and CET-DOX- CMNP-TSLs

- The particle size and zeta potential → dynamic light scattering (DLS)
- Surface morphology of the nanoparticles → TEM
- the magnetic properties of the CMNPs → vibrating sample magnetometer

Measurement of Photo-Thermal Sensitivity of CMNPs in CET-DOX-CMNP-TSLs

- Aqueous suspensions of CET-DOX-CMNP-TSLs were prepared
- 1 mL/sample was loaded into a 96-well microplate.
- Irradiated by NIR at λmax 808 nm with a laser at 2 W/cm2 for 5 min
- Imaged with a thermal imaging camera every 1 min.
- Different concentrations of 20, 50, 100, 200, and 500μg/ mL of CMNP-TSLs.
- The temperature of the sample solution was measured every 1 minute using a thermocouple

Determination of in vitro NIR-Triggered DOX Release

- Release of DOX from the various formulations (DOX-TSLs, DOX-CMNP-TSLs, and CET- DOX-CMNP-TSLs)
- pH 7.4, 6.8, and 5.5
- The influence of pH and NIR irradiation on drug release
- DOX-CMNP-TSLs and CET-DOX-CMNP-TSL
- Treated with NIR (λmax 808 nm, 2 W/cm−2 t=5min
- The same samples without NIR treatment
- UV-Vis spectrophotometer

Assessment of Cellular Uptake of TSLs, CET-TSLs into Breast Cancer Cells

- SKBR-3 (over expressed EGFR) and MCF-7 (low expressed EGFR)
- Studied by flow cytometry
- Fluorescence microscopy
- Both cell lines were seeded in 6-well plates cultured for 24 h
- Culture medium was discarded
- Cells were cleaned 3 times with PBS
- Cells were incubated with 2 mL of serum-free medium
- Containing fluorescent TSLs and CET-TSLs for 2h

Assessment of Cellular Uptake of TSLs, CET-TSLs into Breast Cancer Cells

- Cell culture medium was discarded
- Cells were cleaned three times with cold PBS
- Fluorescence microscope
- Quantification of uptake efficiency by a flow cytometer

Determination of in vitro Cytotoxicity of CET-CMNP-TSLs and CET-DOX-CMNP-TSLs

- The cells were cultured in RPMI 1640 medium
- 10% fetal bovine serum (FBS)
- 1% penicillin/streptomycin
- The cytotoxicity of CET-CMNP-TSLs and CET-DOX- CMNP-TSLs was determined by MTT assays

➢ Assessment of in vivo Photo-Thermal Treatment Efficacy

- Tumor- bearing BALB/C mice
- 1×105 SKBR-3 cells
- Injected **subcutaneously** into the **armpit region**
- Two weeks after the cell injection
- All mice (n=15) were divided into 3 groups
- 200 μL of **normal saline, CMNP- TSLs and CET-CMNP-TSLs**
- The tumors were irradiated with a NIR laser (after 24h)

➢ Assessment of Biosafety of CMNP in Formulations – Hemolysis Assay

- CMNPs, CMNP-TSLs, CET-CMNP-TSLs and CET-CMNP-TSLs+NIR
- Blood samples from healthy rabbits
- Centrifugation at 1000 rpm for 10 min
- washed three times with normal saline
- **Positive and negative controls**, 2% RBCs suspensions were made with **deionized water** and **normal saline** separately
- All samples were diluted with normal saline at a concentration of CMNP at 10, 20, 30, 40 and 50 μg/mL

- Incubated with 2% of RBCs at 37°C for 3 h.
- CET-CMNP-TSLs group was irradiated with NIR radiation at 2 W/cm2 for 5 min
- All treated samples were centrifuged at 1000 rpm for 10 min
- The supernatant from all samples was collected
- The absorbance was measured at **570 nm**


RESULTS

Synthesis and Characterization of CMNPs, TSLs, CMNP-TSLs and CET-DOX-CMNP-TS

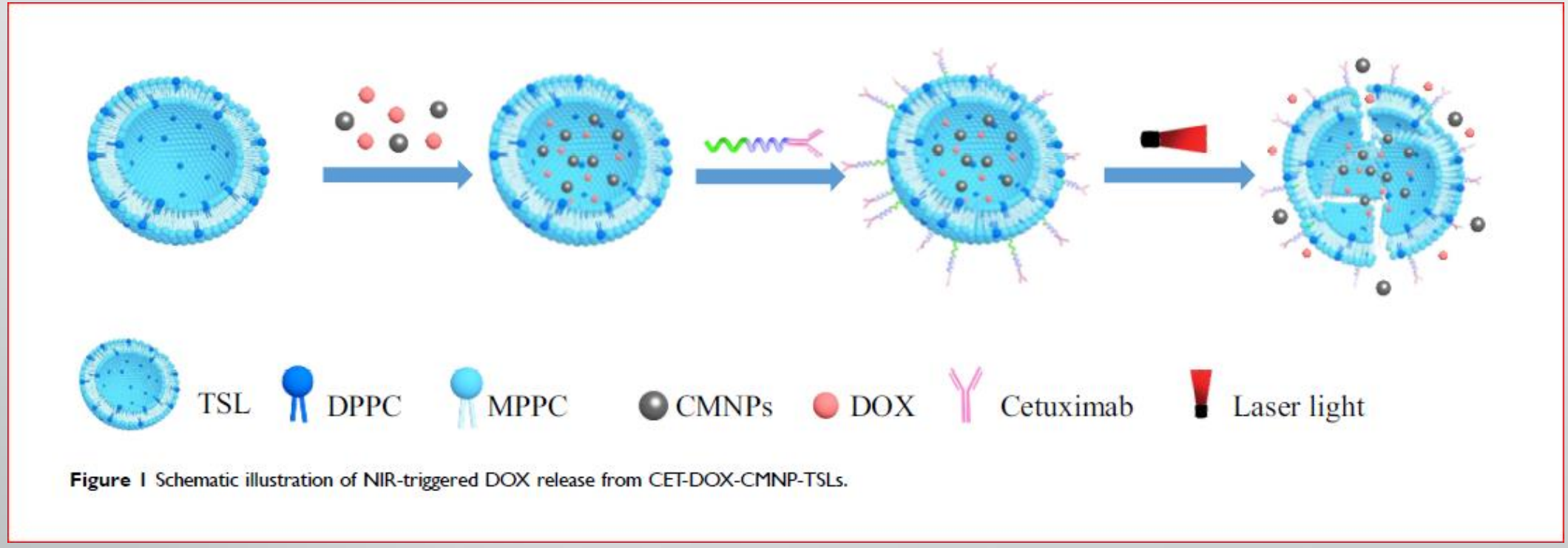


Figure 1 Schematic illustration of NIR-triggered DOX release from CET-DOX-CMNP-TSLs.

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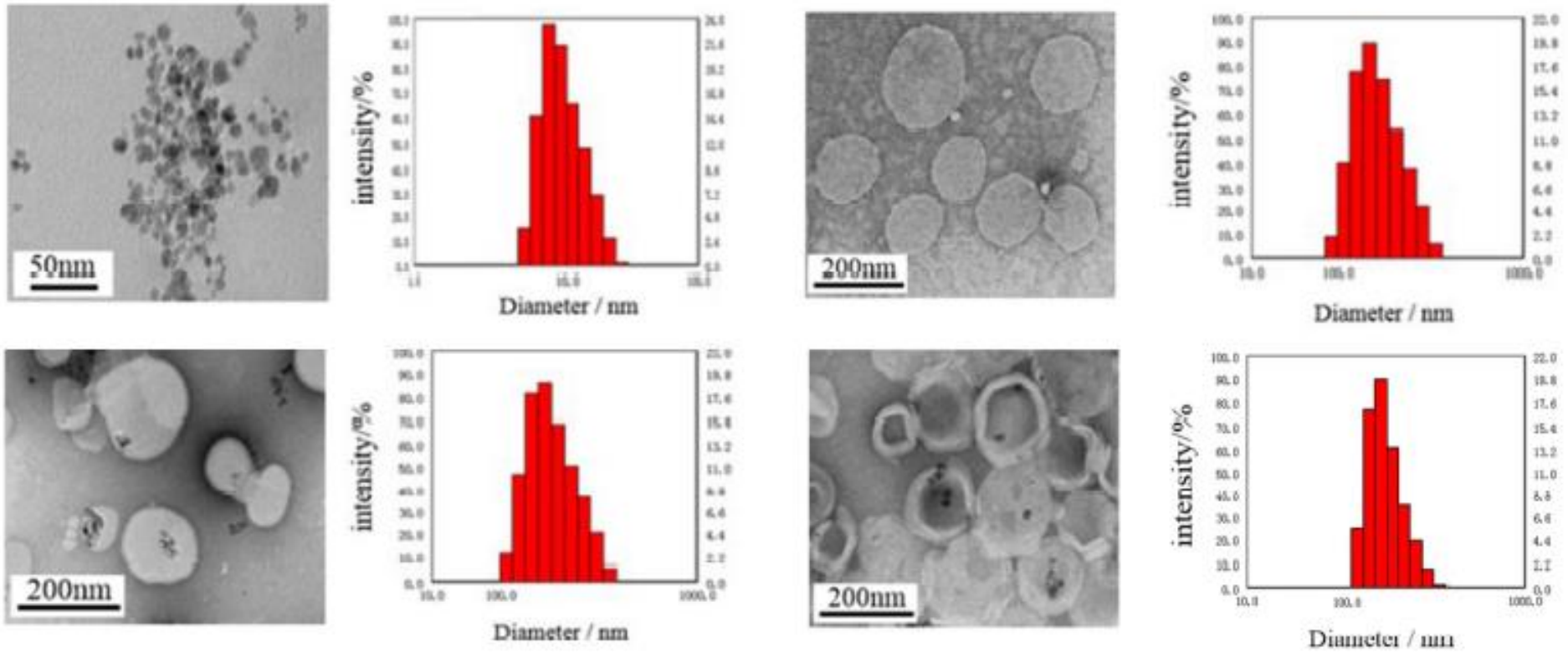


Formulation	Diameter(nm)	Zeta potential(mV)	PDI
CMNPs	8.11 ± 1.12	-26.65 ± 2.27	0.331
TSLs	98.54 ± 2.71	-32.05 ± 1.83	0.211
CMNP-TSLs	101.25 ± 3.38	-29.33 ± 0.54	0.269
CET-DOX-CMNP-TSLs	117.45 ± 3.52	-18.21 ± 1.43	0.138

The magnetization curve of CMNPs as measured by VSM

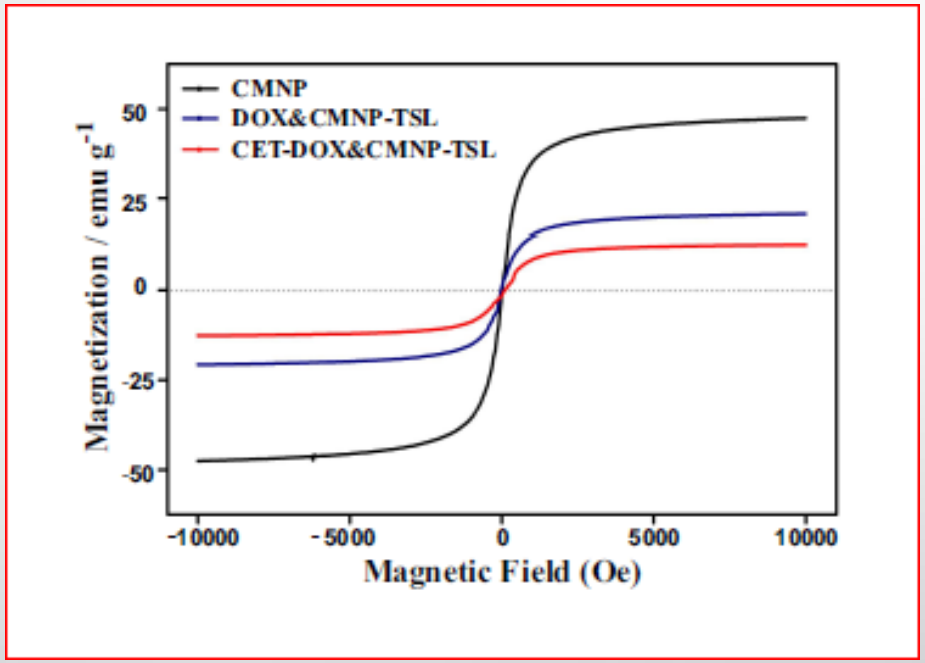
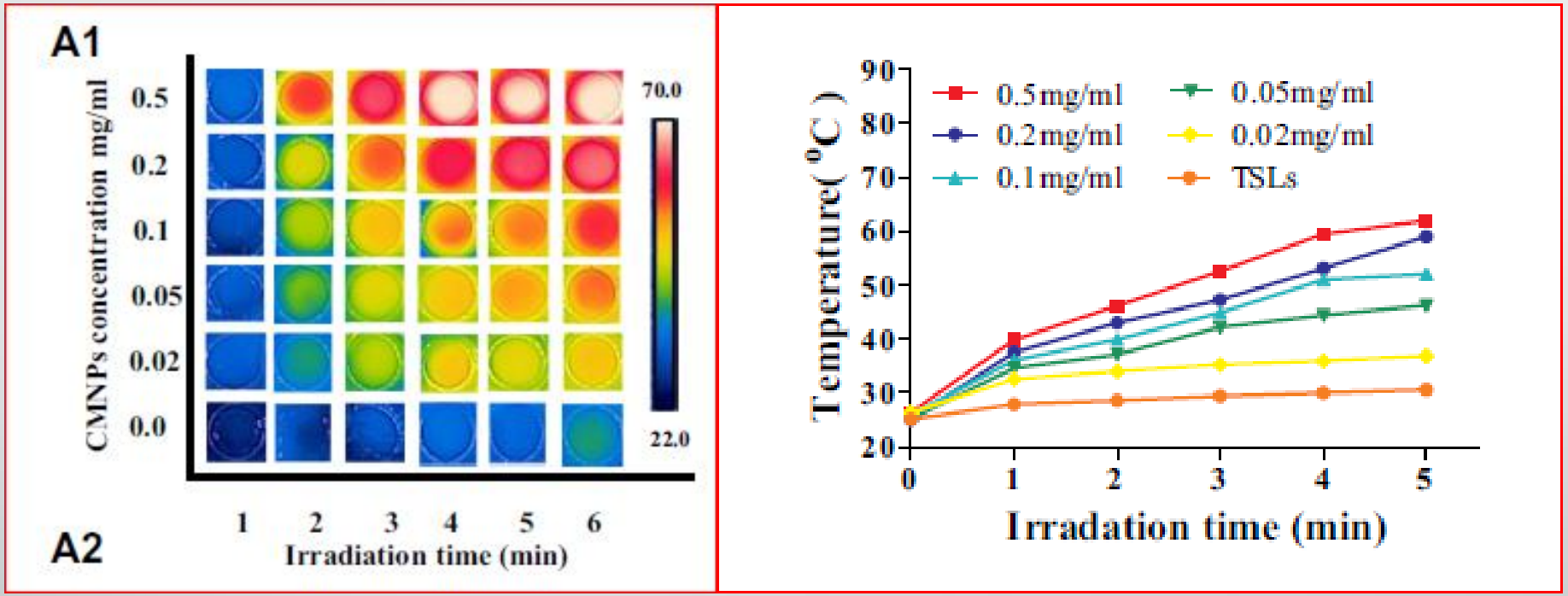
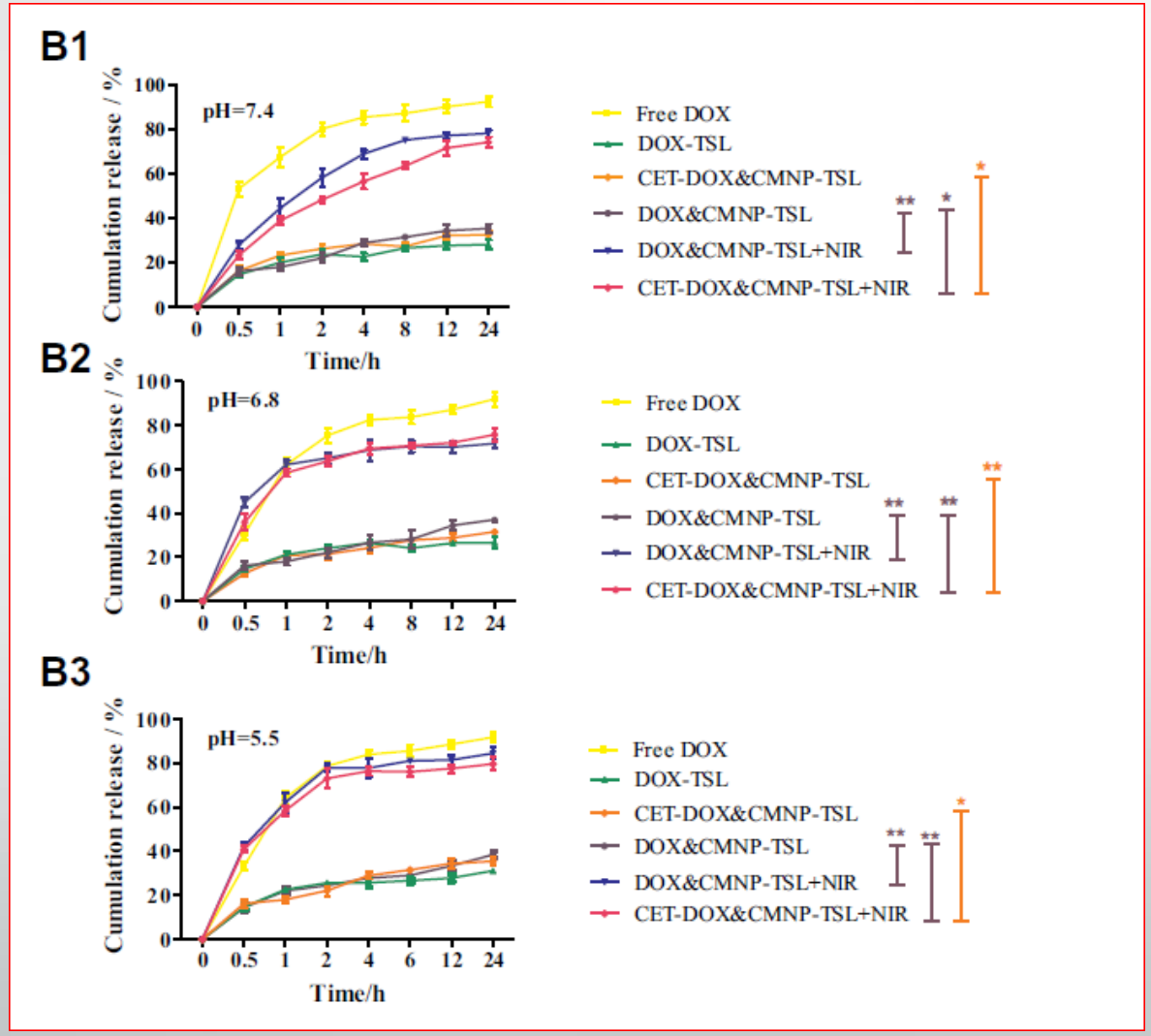


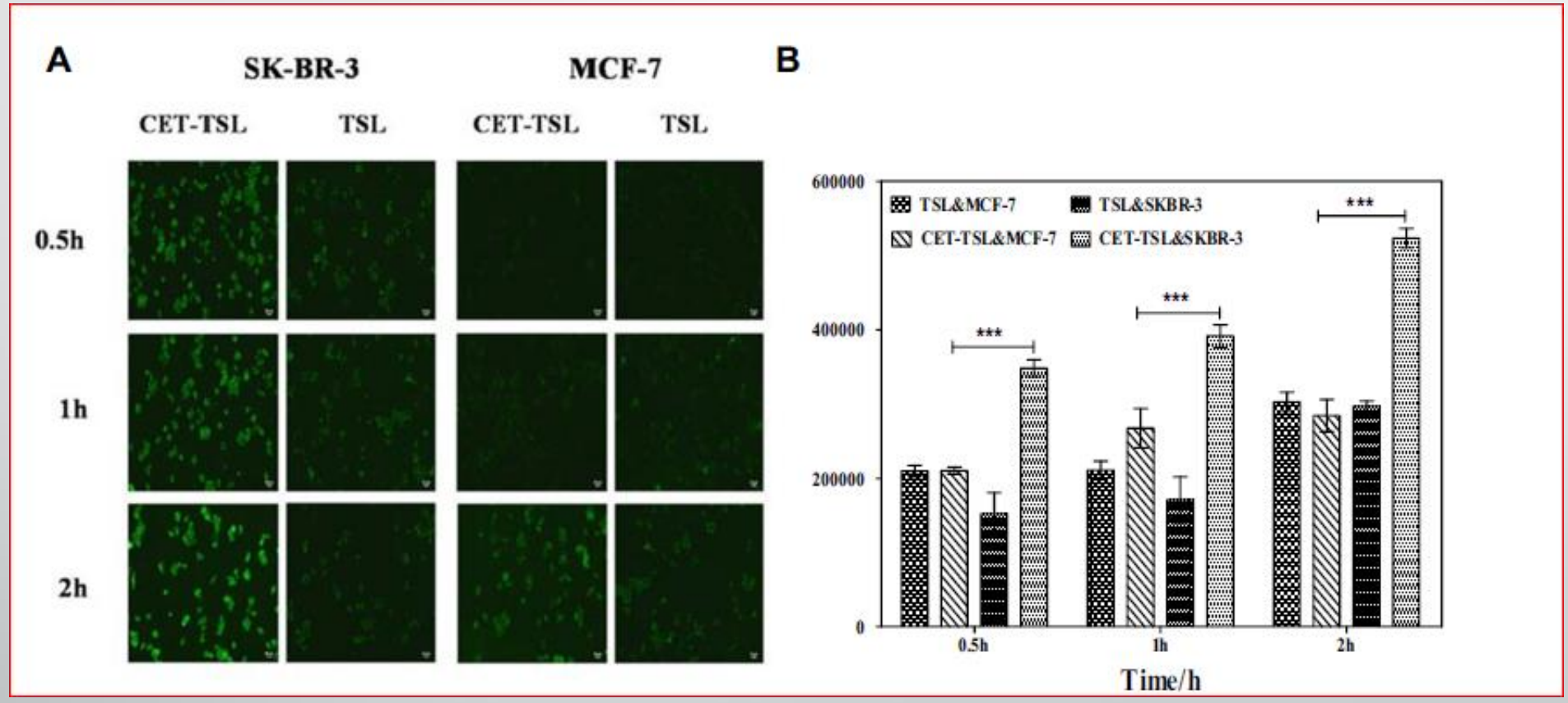
Photo-Thermal Sensitivity Effect of CMNP-TSLs

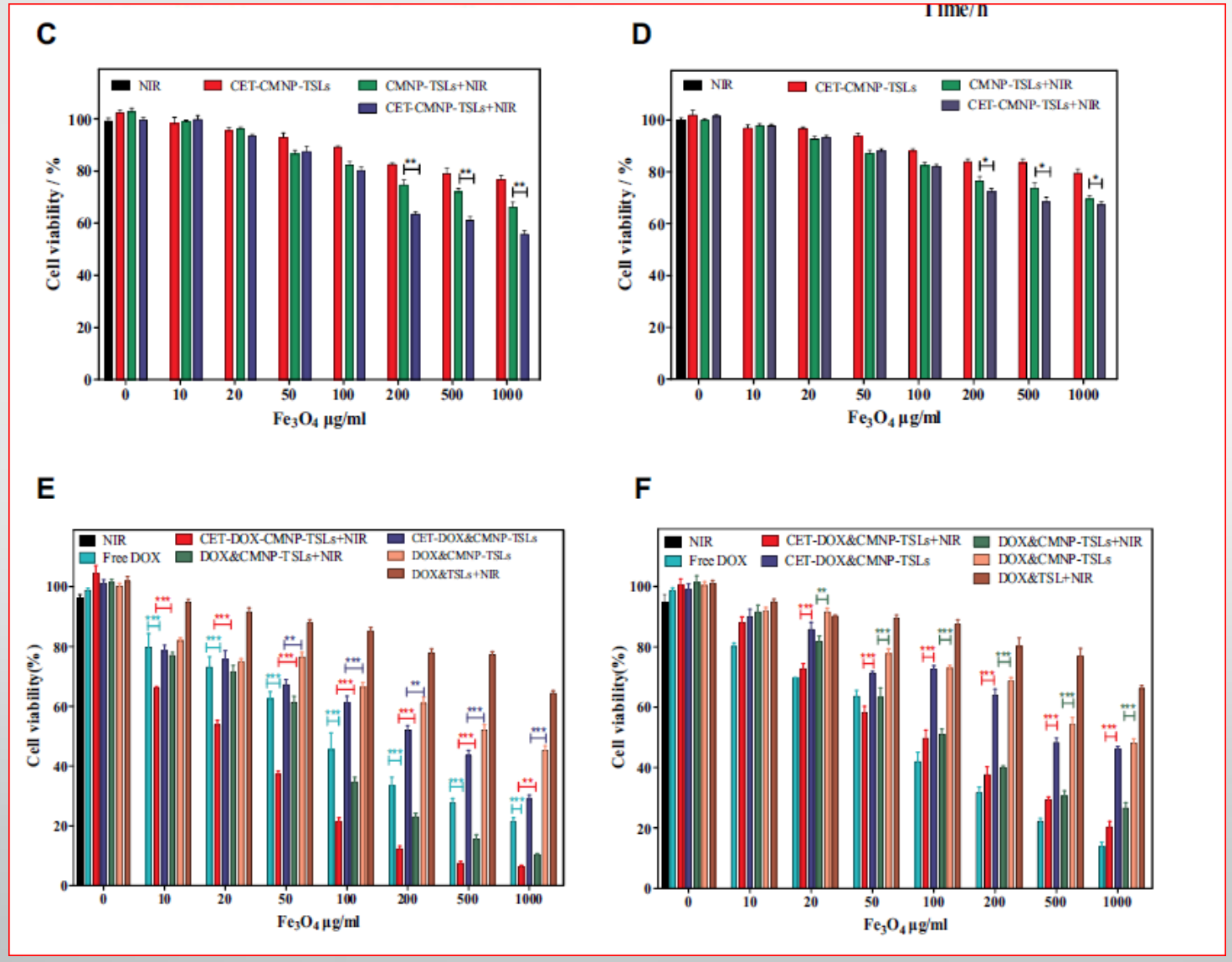


In vitro NIR-Triggered Release of DOX



Breast Cancer Cell Uptake of TSLs and CET-TSLs and Cell Viability Following Treatment with CET-CMNP-TSLs, CET-DOX-CMNP-TSLs




C
NIR
CET-CMNP-TSLs
CMNP-TSLs+NIR
CET-CMNP-TSLs+NIR
Cell viability / %
0
20
40
60
80
100
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10
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50
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200
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Fe3O4 μg/ml
D
NIR
CET-CMNP-TSLs
CMNP-TSLs+NIR
CET-CMNP-TSLs+NIR
Cell viability / %
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40
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100
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10
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Fe3O4 μg/ml
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Free DOX
CET-DOX-CMNP-TSLs+NIR
DOX&CMNP-TSLs+NIR
CET-DOX&CMNP-TSLs
DOX&CMNP-TSLs
DOX&TSLs+NIR
Cell viability(%)
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CET-DOX&CMNP-TSLs+NIR
CET-DOX&CMNP-TSLs
DOX&CMNP-TSLs+NIR
DOX&CMNP-TSLs
DOX&TSL+NIR
Cell viability(%)
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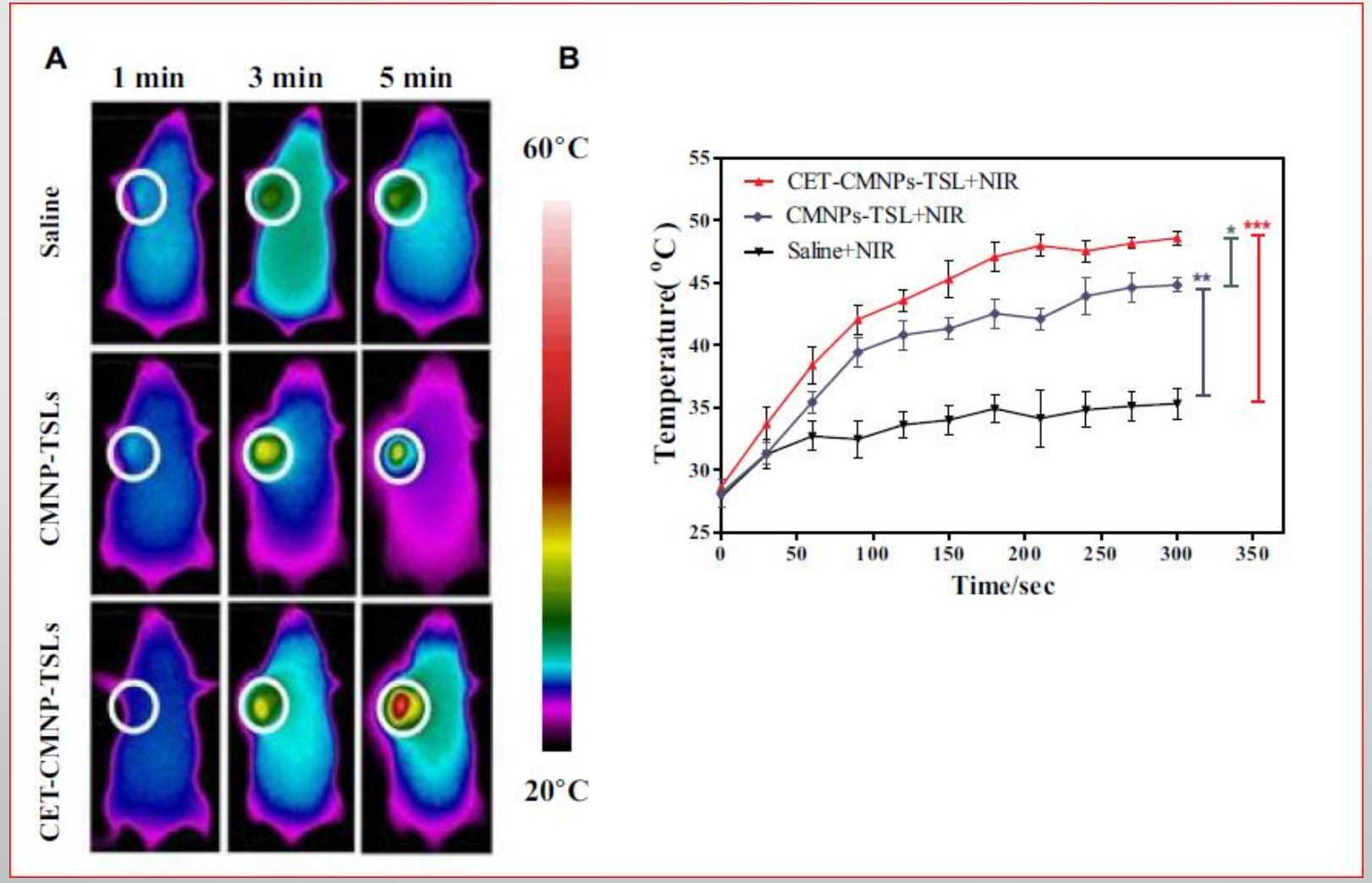
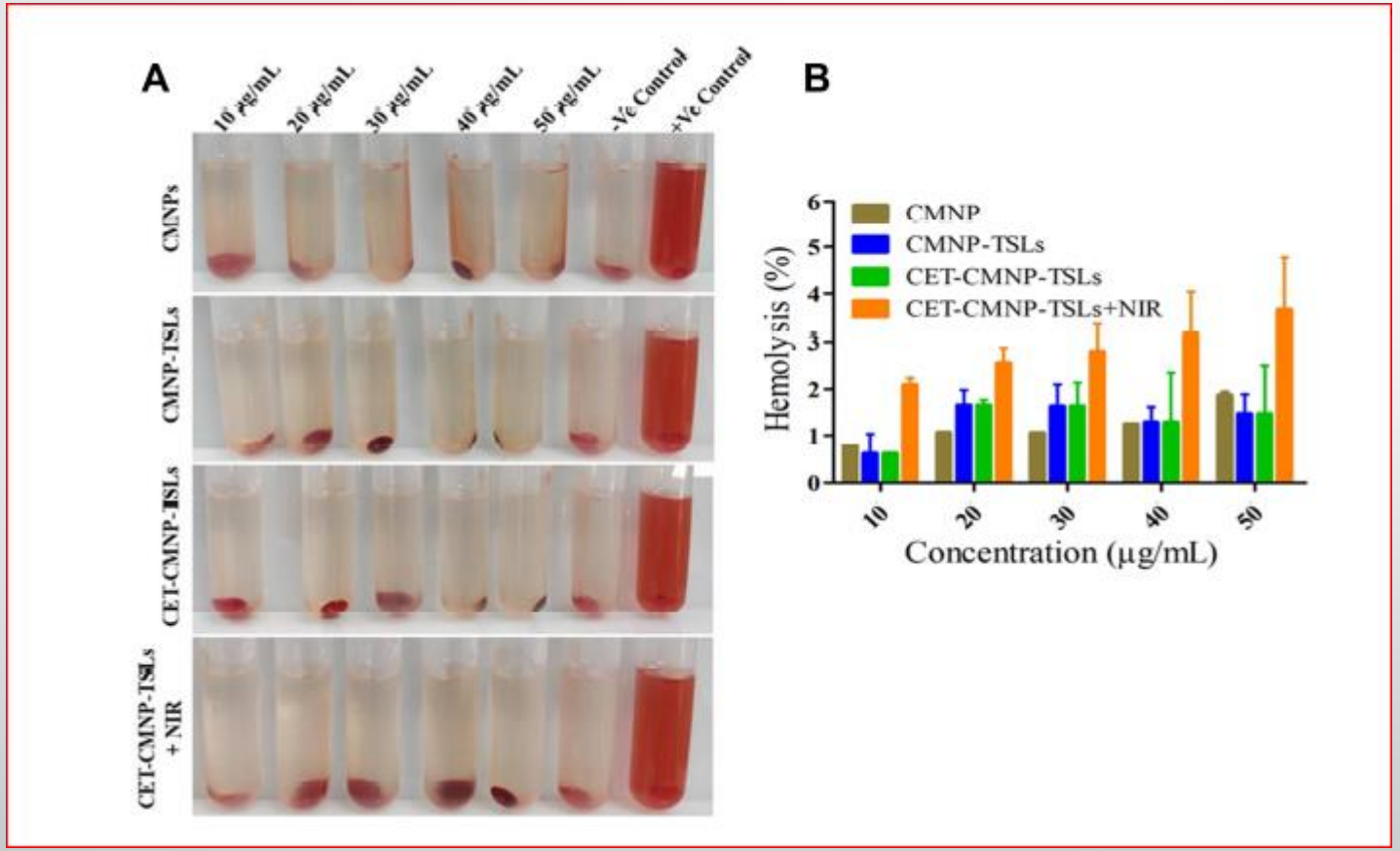
Fe3O4 μg/ml

Photo-Thermal Impact of NIR Laser Irradiation Plus CET-CMNP-TSLs Treatment in Tumor-Bearing Mice



Biosafety Study-Hemolysis Assay



➢ Discussion

- In this study, we designed CET-DOX-CMNP-TSLs when integrated with NIR laser irradiation therapy, enhanced antitumor efficacy.
- **Effective treatments** for many forms of cancer **are still lacking**.
- Smaller nanoparticle size (<200nm) enhances accumulation of the nanoparticles at tumor sites , In our study it was 117±3nm
- DOX and CMNPs loading into the TSLs were enabled by a citric acid coating of MNPs which provides an electrostatic environment
- Similar work was reported by others in which the loading of DOX onto PEGylated-cationic iron oxide was stabilized by citric acid.

- the release of DOX from NIR irradiated samples in acidic media at pH 6.8 and 5.5 was comparatively higher than at pH 7.4 and non-irradiated samples
- The pH in the tumor microenvironment is weakly acidic , the released DOX in tumors is more than in normal tissues
- Cancer cells express various types of receptors, with which a specific antibody can bind to facilitate selective antibody-targeted therapeutic effects; these include folate, integrin, and EGFR
- various researchers have demonstrated the selective internalization of monoclonal antibody-modified nanoparticles in comparison to non-modified nanoparticles

- Studies by others and us have demonstrated the selective binding of CET with EGFR expressed on breast cancer cells
- the uptake of CET-TSLs was greater in comparison with the uptake of TSLs in SKBR-3 cell lines than in MCF-7 cell; similar results have been reported by others
- combination therapies have often shown better, efficacy in comparison to monotherapies
- combination of chemotherapeutic and photo-thermal therapy should be considered as a promising strategy to enhance the anti-cancer impact of chemotherapeutics

- Iron-based nanoparticles, have been explored as a possible nanocarrier system for various anti-cancer drugs
- To exploit the thermal and magnetic properties of iron oxide, we prepared CMNP and DOX-loaded TSLs that were coated with CET to target EGFR-expressing breast cancer cells
- Our results showed that breast cancer cell viability significantly declined when NIR radiation was added to cells treated with the chemotherapeutic nanocarrier.
- Free DOX was also able to decrease breast cancer cell viability to a similar but much higher doses were required

- NIR laser irradiation could be combined with chemotherapeutic nanocarriers to reduce the dose of a chemotherapeutic drug required for treatment efficacy
- the tumor surface temperature found during NIR laser irradiated mice treated with CET-CMNP-TSLs or CMNP-TSLs, was substantially greater in comparison to the mice treated with normal saline
- hemolysis assays showed that these nanocarriers are biocompatible and safe

➢ Conclusion

- we have developed a novel and selective chemotherapeutic nanocarrier system
- facilitates drug delivery to EGFR-expressing breast cancer cells enhancing the impact of photo-thermal therapy
- These CET-DOX-CMNP-TSLs reduced breast cancer cell viability and increased tumor temperature when combined with NIR laser irradiation
- iron oxide MNPs is promising nanomaterial for photothermal tumor therapy
- iron oxide could be more easily degraded and metabolized in the body
- iron oxide MNPs encapsulating by TSLcould achieve NIR-triggered drug release behavior which will demonstrate desirable photothermal therapeutic efficiency


thank you